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Aberrant Expression of *HOXA9*, *DEK*, *CBL* and *CSF1R* in Acute Myeloid Leukemia

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Previous gene function analyses have indicated that *HOXA9*, *DEK*, *CBL* and *CSF1R* are aberrantly expressed in acute myeloid leukemia (AML). We analyzed the expression of these genes in a series of 41 adult patients with AML using quantitative real-time RT-PCR, and tested the association of the expression with the following hematologic and clinical parameters: age, FAB, immunophenotype and karyotype aberrations. A high proportion of the patients showed over- or underexpression of the analyzed genes. *DEK* was overexpressed in 98% of the cases, whereas *CBL*, *CSF1R* and *HOXA9* were either overexpressed in 20%, 17% and 78% or underexpressed in 20%, 42% and 15% of the cases, respectively. Patients whose karyotype contained t(8;21)(q22;q22), showed lower relative expression of *HOXA9* at a statistically significant level ($p < 0.05$). Bone marrow samples without expression of CD34 antigen were associated with either overexpression of *DEK* or *HOXA9*. Furthermore, an association was found between the AML-M2 subtype and lower expression of *CBL*, *CSF1R* or *HOXA9*, and between the AML-M5 subtype and *CBL* or *CSF1R* overexpression.

Keywords: Quantitative real-time RT-PCR; Acute myeloid leukemia; Gene expression; Array-based methods

INTRODUCTION

The array-based methods have revealed a great amount of new information of genes expressed in acute myeloid leukemia (AML) [1,2]. In this study, we selected four genes that were previously detected to be aberrantly expressed in AML by array techniques: homeobox protein hox-a9 (*HOXA9*), dek oncogene (*DEK*), macrophage colony stimulating factor I receptor precursor (*CSF1R*) and c-cbl protooncogene (*CBL*) (Table I).

HOXA9 is a transcriptional factor potentially associated in myeloid differentiation. The chromosomal translocation t(7;11)(p15;p15), which involves a fusion between *HOXA9* and the nucleoporin *NUP98* resulting in the inhibition function of *HOXA9*, is present in a small subset of AML patients with poor prognosis [3,4].

DEK is a nuclear protein, with both RNA and DNA binding properties, expressed in many tissues [5,6]. This gene is implicated in a subset of AML carrying the t(6;9)(p23;q34) in which the DEK-CAN protein confers poor prognosis [7,8].

CBL protooncogene product participates in signal transduction of hematopoietic cells [9]. *CBL* generates negative regulation of CSF-1 by binding and stimulating *CSF1R* ubiquitination, which increases the endocytic rate of *CSF1R* and reduces the signal of activated receptors on cell membrane [10].

The expression of the four genes in 41 AML patients at diagnosis was studied using quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR). Furthermore, we analyzed whether aberrant expression of these genes was associated with hematologic, cytogenetic or clinical parameters.

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TABLE I Features of genes included in quantitative expression analysis

Genes	mRNA Gene Bank accession number	Chromosomal location
Blood disorder proteins		
DEK protein	X64229	6p23
Oncogenes and protein kinases activated in hematopoietic cells		
C-cbl protooncogene	X57110	11q23.3
Macrophage colony stimulating factor I receptor precursor (*CSF-1-R*)	X03663	5q33.3-35
Transcriptor factor		
Homeobox protein hox-a9 (*HOXA9*)	U82759	7p15-p14
Housekeeping gene		
Cytoplasmatic beta-actin (*ACTB*)	X00351	7p15-p12

MATERIALS AND METHODS

Patients

Bone marrow samples were obtained from 41 adult patients with AML at the time of diagnosis. Fourteen of the 41 patients (patients 1–14) belonged to the AML-99 protocol of the Catalan Group of Study and Treatment of AML (CETLAM). The remaining 27 patients (patients 15–41) were diagnosed and treated according to the protocol of the Department of Medicine, Helsinki University Central Hospital. Patients nos 15–29 had previously undergone cDNA array analysis as reported by Larramendy *et al.* [2] Table II summarizes the laboratory and clinical data of the patients. Two bone marrow aspirates from healthy donors were included as controls.

RNA Extraction

Total RNA was extracted from bone marrow samples according to standard protocol, as described previously [2]. DNase treatment of total RNA was performed according to the Atlas cDNA expression array's user manual (Clontech Laboratories Inc., Palo Alto, CA, USA). The quality and the integrity of the RNA were checked on 1% agarose gel after electrophoresis.

cDNA Synthesis

cDNA synthesis was performed from total RNA by using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Real-time Quantitative RT-PCR

The oligonucleotide primers were designed and produced by TIB MolBiol (Berlin, Germany) (Table III). Primers were tested *versus* the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) to confirm the specific amplified product.

Quantitative gene expression analysis of four AML-related genes and one housekeeping gene, beta-actin (*ACTB*) was performed. QRT-PCR was carried out using LightCycler FastStart DNA Master SYBR Green I and LightCycler Instrument (Roche), as described previously [2]. Four dilutions of beta-globulin gene (DNA Control kit; Roche), from 0.015 to 15 ng/μl, were used to acquire the standard curve in each experiment. In every assay, runs were independently repeated two times and a negative control was included. The amplification of PCR products was verified using the melting curve analysis option. Data analysis was performed using the LightCycler Software Version 3.5.

The housekeeping gene *ACTB* was used as reference against which the expression level of the transcript genes of interest was normalized. For every sample, the target mRNA copy number was divided by the *ACTB* mRNA copy number to obtain a normalized target/*ACTB* value. This value was then divided by the average normalized target/*ACTB* value of the normal bone marrow samples to obtain the relative target value [11]. Relative expression levels higher than 3 were considered to indicate overexpression, and lower than 0.3 underexpression.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows (Version 10.0). After the normalization of expression values, the Kaplan–Meier analysis was used to estimate survival distributions, and log-rank test to evaluate significant differences among survival curves [12]. Clinical and biological parameters were compared using the Mann–Whitney *U* Test. The chosen level of significance was 0.05, unless otherwise indicated.

RESULTS

HOXA9, *DEK*, *CBL* and *CSF1R* quantification data obtained using the LightCycler software were normalized using the *ACTB* housekeeping gene. Gene expression in the AML patients was compared to that in the normal bone marrow samples (Table IV).

TABLE II Biological and clinical data of 41 AML patients

	Sex	Age	Date of diagnosis	FAB	Karyotype	Immunophenotype	Blasts in BM (%)	WBC (× 109/l)
1	M	34	9/05/01	M4	46,XY	CD13+,CD33+,CD34+,CD56−,CD64−, CD14−,CD7−,CD19−,CD2−,CD117+	43	1.6
2	F	51	12/06/01	M4	46,XX,t(3;12)(q21;q24),t(8;21)(q22;q22)/46,XX	CD13+,CD33+,CD34+,CD56+,CD64−, CD14−,CD7−,CD19−,CD2−,CD117+	74	18
3	M	58	29/06/01	M5b	46,XY	CD13+,CD33+,CD34−,CD56−,CD64−, CD14−,CD7−,CD19−,CD2−,CD117+	96	14.6
4	F	57	13/07/01	M5b	46,XX	CD13+,CD33+,CD34+,CD56−,CD64+, CD14−,CD7−,CD19+,CD2−,CD117+	60	31.2
5	F	60	9/08/01	M5b	ND	CD13+,CD33+,CD34+,CD56+,CD64+, CD14+,CD7+,CD19−,CD2−,CD117+	44	26
6	F	26	20/08/01	M2	48,XX,t(8;21)(q22;q22),del(11)(q21q23),+15,+21c	CD13+,CD33+,CD34+,CD56−,CD64−,CD14−, CD7−,CD19−,CD2−,CD117+	60	94
7	M	53	22/08/01	M5	45,XY,t(5;19)(q11;p12),der(7)t(7;?)(p15;?), der(12)t(12;18)(p?;q?), del(16)(q13),−17,der(18)t(17;18)(q21;q?),dup(22)(q?)	CD13+,CD33+,CD34+,CD56−,CD64+, CD14+,CD7−,CD19+,CD2−,CD117+	76	61
8	F	58	4/09/01	M5b	46,XX	CD13+,CD33+,CD34+,CD56+,CD64+, CD14+,CD7−,CD19+,CD2−,CD117+	52	37.8
9	F	40	9/10/01	M2	46,XX	CD13−,CD33+,CD34+,CD56+,CD64−, CD14−,CD7+,CD19−,CD2−,CD117+	30	12
10	F	36	17/10/01	M1	47,XX,+4	CD13+,CD33+,CD34−,CD56−,CD64−, CD14−,CD7−,CD19−,CD2−,CD117+	65	28.6
11	F	59	22/10/01	M2	ND	CD13+,CD33+,CD34+,CD56−,CD64−, CD14−,CD7−,CD19−,CD2−,CD117+	60	1.26
12	M	38	4/11/01	M1	47,XXYc	CD13+,CD33+,CD34+,CD56−,CD64+, CD14−,CD7+,CD19+,CD2+,CD117+	70	5
13	M	35	2/11/01	M5	45,XY,−7	CD13+,CD33+,CD34+,CD56−,CD64−, CD14−,CD7−,CD19−,CD2−,CD117+	92	73
14	F	46	1/11/01	M2	46,XX,inv(11)(p15q23)	CD13+,CD33+,CD34+,CD56−,CD64−, CD14−,CD7−,CD19−,CD2−,CD117+	58	110
15	F	50	5/06/99	M2	46,XX,t(8;21)(q22;q22)	CD13+,CD33+,CD34+,CD56−,CD64+	50	105.2
16	M	21	2/11/98	M4	46,XY	CD7+,CD13+,CD14−,CD33+,CD34+,CD56−,CD64+	90	6.9
17	M	66	12/03/99	M4	46,XY,inv(16)(p13q22)	CD13+,CD14+,CD33+,CD34+,CD56−,CD64+	50	85.8
18	F	30	18/09/98	M1	46,XX	CD13+,CD33+,CD34+,CD56−,CD64−	91	43.3
19	F	61	13/03/98	M2	46,XX	CD13−,CD33+,CD34+,CD34−,CD56+	75	80.9
20	F	52	30/01/98	M5	46,XX	CD13+,CD14+,CD33+,CD34−,CD56+,CD64+	85	94.4
21	F	46	18/08/99	M4	46,XX	CD7+,CD13+,CD19+,CD33+,CD34+,CD56−,CD64+	50	185.7
22	M	51	16/07/99	M2	46,XX,t(8;21)(q22;q22)	CD13+,CD19+,CD33+,CD34+	40	31
23	F	65	5/05/99	M2	45,XX,dup(1)(p?),del(5q),t(14;19),−17,inc*	CD13+,CD33+,CD34+,CD56−,CD64+	40	2.2
24	M	67	9/09/99	M2	46,XY	CD13+,CD14−,CD33+,CD34−,CD56−,CD64−	60	75.1
25	M	52	11/10/99	M2	45,X,-Y,t(8;21)(q22;q22)	CD13+,CD33+,CD34+	80	4.6
26	M	60	3/03/00	M1	46,XY	CD2+,CD7+,CD13+,CD33+,CD34+,CD117+	90	132
27	F	66	4/02/00	M2	46,XX	CD13+,CD33−,CD34−,CD56+	80	206
28	M	53	29/12/99	M2	45,X,-Y,t(8;21)(q22;q22)	CD13+,CD14−,CD33+,CD56+,CD64+,CD34+	90	8.6
29	M	65	9/06/00	M4	47,XY,del(9q?),+8/46,XY,del(9q?)/46,XY	CD33+,CD34−,CD56+,CD64+	80	37.5
30	F	72	29/06/00	M1	46,XX	CD13+,CD14−,CD33+,CD56+,CD64−,CD117+,CD34−	90	158
31	M	50	19/04/00	M5	46,XY	CD13+,CD14+,CD33+,CD34−,CD56+,CD64+	95	58.5
32	M	44	12/09/00	M2	46,XY,inv(11)(p15q23)	CD13+,CD33+,CD34−,CD56−,CD64−,CD117+	45	2.7
33	F	35	1/09/00	M3	46,XX,t(15;17)(q22;q11-12)	CD13+,CD33+,CD34−,CD56−,CD117+	90	1.2

TABLE II – *continued*

	Sex	Age	Date of diagnosis	FAB	Karyotype	Immunophenotype	Blasts in BM (%)	WBC (× 109/l)
34	F	26	14/02/00	M3	46,XX	CD13+,CD33+,CD34−	75	2.4
35	M	23	20/08/99	M2	45,X,-Y,t(8;21)(q22;q22)	CD13+,CD33+,CD34+,CD56+,CD64+	65	41.4
36	F	51	3/11/00	M2	46,XX,t(3;10),t(7;15)*	CD13+,CD33+,CD34+,CD56−,CD64−,CD117+	70	3.4
37	M	61	8/12/99	M4	46,XY	CD13+,CD14+,CD33+,CD34+,CD56+,CD64+	90	80.2
38	F	68	13/12/99	M5	46,XX	CD13+,CD14+,CD33+,CD34−,CD56+,CD64+	50	100.6
39	F	51	2/02/00	M4	46,XX	CD13+,CD14+,CD33+,CD34−,CD56−,CD64+	35	10.4
40	F	67	24/03/00	M0	45,XX,−3,del(5)(q?21),add(7)(q?32),−10, del(12)(q22),add(18)(p11),+2mar	CD7+,CD13+,CD33+,CD34−,CD56+,CD64−,CD117+	70	1.3
41	M	48	30/12/99	M2	46,XY	CD13+,CD33+,CD34+,CD56−	35	5.5

M, male; F, female; WBC, white blood cell count; ND, not done; BM, bone marrow.
* Karyotype described by multicolor *in situ* hybridization.

HOXA9 was overexpressed in 32 cases (78%) and underexpressed in 6 patients (14.6%), whereas no altered expression was observed in the remaining 3 patients (7.3%). All but one of the patients (97.6%) showed overexpression of *DEK*. Overexpression of *CSF1R* was seen in 7 patients (17.1%), underexpression in 17 samples (41.5%) and normal expression in 17 patients (41.5%). *CBL* was overexpressed in 8 patients (19.5%) and underexpressed in 8 patients (19.5%), whereas no altered expression was observed in the remaining 25 patients (61%).

Patients with t(8;21)(q22;q22) showed decreased expression of *HOXA9* ($p = 0.004$). An association was found between CD34− bone marrow samples and overexpression of *DEK* ($p = 0.01$) and *HOXA9* ($p = 0.005$). When FAB subtypes were considered, M2 was associated with lower expression levels of *CBL* ($p = 0.034$), *CSF1R* ($p = 0.034$) and *HOXA9* (0.045). In the M5 subtype, both *CBL* and *CSF1R* were overexpressed ($p = 0.012$ and $p = 0.021$, respectively). No further association was seen between gene expression and clinical-pathological parameters (Table V). Nevertheless, statistically significant associations were observed between decreased expression of *CBL* and age older than 60 years ($p = 0.034$).

DISCUSSION

Gene expression profiles of *HOXA9*, *DEK*, *CSF1R* and *CBL* studied by real-time QRT-PCR showed expression changes in 92.7%, 97.6%, 58.5% and 39% of the cases, respectively, confirming previous cDNA microarray results [1,2].

Overexpression of *HOXA9* transcript was observed in nearly 80% of cases. This result verifies several previous findings demonstrating enhanced expression of *HOXA9* in bone marrow AML samples [1,13–15]. As overexpression of the gene does not seem to be present in acute lymphoblastic leukemia [16], it may be considered as a genetic marker for AML. Detection of *HOXA9* overexpression was an independent event from the presence of t(7;11)(p15;p15), since none of the 41 patients included in the analysis showed this chromosomal rearrangement.

The present results reveal the overexpression of *DEK* transcripts in the majority of AML cases (97.6%). This finding confirms our previous results by cDNA array [2]. The results from the present study highlight the potential role of *DEK* as a genetic marker in the initiation and progression of AML. As none of the patients presented t(6;9)(p23;q34) in their karyotype, the overexpression of the gene resulted from a mechanism other than translocation, as recently proposed by Larramendy *et al.* [2].

Underexpression of *CBL* or overexpression of *CSF1R* increases the ratio of activated receptors on cell membrane [10]. Either under- or overexpression of

TABLE III Primers

Gene	Sequence 5′-3′ F R	Position	Length (pb)	CG (%)	Tm (°C)	Product size (bp)
ACTB	AgCCTCgCCTTTgCCgA	23F	17	64.7	68.5	174
	CTggTgCCTggggCg	196R	15	80.0	64.1	
DEK	gTgggTCAgTTCAgTggC	412F	18	61.1	61.9	291
	AggACATTTggTTCgCTTAg	702R	20	45.0	60.7	
CSF1R	CACCAAgCTCgCAATCCCTC	1015F	20	60.0	63.7	169
	CTCTACCACCCggAAgAACA	1183R	20	55.5	63.9	
CBL	ATgTCCCAAAgCCACCTg	2472F	18	55.6	63.6	217
	gCAggACCACTACCTTgCT	2688R	19	57.9	61.9	
HOX9A	AAAAATCTACCTgTTCCTgAC	1174F	21	38.1	56.3	236
	CTATCTTCCACAATCACAATggg	1409F	23	43.5	64.1	

F, forward; R, reverse; Tm, melting temperature.

CBL or *CSF1R* was seen in 34% of the patients. This group of patients may have an increased proliferation signal to CSF1 stimulation [17]. An association was observed between lower expression levels of *CBL* and patient age older than 60 years. The increased expression levels of *CBL* or *CSF1R* in the M5 subtype, the monocytic AML, is interesting as their expression is normal in the myelomonocytic AML and decreased in the M2 subtype.

Sauvageau *et al.* [18] demonstrated for the first time that homeobox A genes are expressed in human CD34+ cells and underexpressed in CD34− cells in normal bone marrow samples. Kawagoe *et al.* [13] found consistent results in normal bone marrow samples, but no difference in *HOXA9* expression between CD34+ and CD34− cells was detected in AML bone marrow samples. According to these previous reports, the present observation of higher *HOXA9* overexpression in CD34− AML samples than in CD34+ AML samples may indicate deregulation of *HOXA9* expression during leukemogenesis. In contrast, little is known about the phenotypic expression of *DEK* in myeloid cells. Our CD34− AML samples showed higher *DEK* expression than CD34+ AML samples, providing a clue to understand its patterns of activation during leukemic cell differentiation.

Lower relative amounts of *HOXA9* were detected in patients with t(8;21)(q22;q22) ($p = 0.004$). Similarly, Drabkin *et al.* [15] detected low homeobox (*HOX*) gene expression in cases with prognostically favorable cytogenetic features. This association suggests that oncogenic proteins produced by a chromosomal translocation, such us AML1-ETO in t(8;21)(q22;q22), affect *HOX* gene expression, altering the transcription function and disrupting myeloid cell differentiation at different linages [19].

Although *HOXA9* overexpression has been shown to correlate with treatment failure in AML [1], extended studies have not been able to reveal any significant associations with complete response rates or event-free survival [15]. Whether the expression status of *HOXA9* in our patients has any prognostic value is an issue that we will study once the follow-up time is long enough to provide statistical power to the analysis.

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TABLE IV QRT-PCR results as relative expression level

Relative expression level*	DEK *n* (%)	CBL *n* (%)	CSF1R *n* (%)	HOXA9 *n* (%)
Overexpression	40 (97.6)	8 (19.5)	7 (17.1)	32 (78)
3 to 10	2 (4.9)	6 (14.6)	4 (9.7)	2 (4.9)
11 to 100	14 (34.1)	2 (4.9)	1 (2.4)	4 (9.7)
101 to 1000	13 (31.7)	–	2 (4.9)	15 (36.6)
1001 to 10000	10 (24.4)	–	–	6 (14.6)
10001 to 100000	1 (2.4)	–	–	6 (14.6)
> 100000	–	–	–	1 (2.4)
Normal expression	1 (2.4)	25 (61)	17 (41.5)	3 (7.3)
Underexpression	–	8 (19.5)	17 (41.5)	6 (14.6)
0.3 to 0.1	–	6 (14.6)	12 (29.3)	2 (4.9)
0.09 to 0.01	–	2 (4.9)	4 (9.7)	2 (4.9)
0.009 to 0.001	–	–	1 (2.4)	2 (4.9)

Bold type, high frequencies for each gene.
* Relative expression level >3 was considered to indicate overexpression and <0.3 underexpression.

TABLE V Relationship between relative gene expression level and standard clinical and biological factors

Grouping variable		*n* (%)	*DEK**	$p^{\dagger}$	*HOXA9**	$p^{\dagger}$	*CBL**	$p^{\dagger}$	*CSF1R**	$p^{\dagger}$
Age (years)	≤60	31 (75.6)	1697.5 ± 1287.7	NS	3372 ± 4851.4	NS	4.9 ± 5.5	0.034	9.8 ± 11.9	NS
	>60	10 (24.4)	608.8 ± 692.1		1848 ± 2707.9		0.7 ± 0.7		0.5 ± 0.5	
WBC	$<30\times10^9$/l	22 (53.6)	1800.4 ± 1678.3	NS	4635.9 ± 6905	NS	6.1 ± 7.83	NS	13.1 ± 5.8	NS
	$\geq30\times10^9$/l	19 (46.3)	1005.4 ± 989.8		1106.4 ± 1346.4		1.3 ± 0.77		1.1 ± 0.9	
Blasts in BM	≤70%	23 (56)	777.1 ± 505.1	NS	1356.3 ± 2035.1	NS	1.8 ± 1.2	NS	7.1 ± 11.6	NS
	>70%	18 (44)	2229.2 ± 2020.9		5100.8 ± 7673.4		6.5 ± 9		8.1 ± 13.5	
Karyotype	Normal	21 (51.2)	1756.1 ± 1779.1	NS	3693.9 ± 6630.8	NS	5.5 ± 7.8	NS	6.9 ± 11.7	NS
	Abnormal	18 (43.9)	1155.8 ± 805.2		1020.7 ± 1506.3		1.3 ± 0.8		1.2 ± 1	
Karyotype t(8;21)(q22;q22)	Absent	34 (82.9)	1555.1 ± 1182.9	NS	3612.5 ± 4464.5	0.004	4.5 ± 5	NS	9 ± 10.9	NS
	Present	7 (17.1)	833.8 ± 1189.6		26.9 ± 48.7		0.9 ± 0.7		0.4 ± 0.4	
Immunophenotype	CD56+	15 (36.6)	1299.8 ± 1154.5	NS	2868 ± 3691	NS	1.7 ± 1.8	NS	9.5 ± 19.7	NS
	CD56 −	22 (53.6)	754.7 ± 585		3575.9 ± 6637		5.68 ± 7.8		7.58 ± 11.7	
	CD34+	25 (61)	567.2 ± 466.3	0.01	1199.3 ± 1975.6	0.005	2.4 ± 1.3	NS	7 ± 11.2	NS
	CD34 −	16 (39)	2783.1 ± 2408.6		5814.4 ± 9353.4		6.2 ± 11.1		8.3 ± 13.2	
	CD64+	18 (44)	1050.1 ± 991.2	NS	2258 ± 3062.1	NS	2.4 ± 1.7	NS	9.1 ± 15.8	NS
	CD64 −	15 (36.6)	750.2 ± 771.2		5137 ± 10054.8		7.1 ± 21.4		9.5 ± 17.7	
FAB	M1	5 (12.2)	1052.8 ± 2432	NS	562.6 ± 712.7	NS	2.5 ± 3.2	NS	0.97 ± 1.5	NS
	Other subtypes	36 (87.8)	1484.6 ± 1098.4		3338.8 ± 4181.4		4.1 ± 4.7		8.4 ± 10.2	
	M2	16 (39)	935 ± 742.1	NS	388.7 ± 413.9	0.045	0.8 ± 0.38	0.034	0.5 ± 0.5	0.034
	Other subtypes	25 (61)	1750 ± 1575.4		4617.7 ± 6072.3		5.8 ± 6.9		12 ± 14.8	
	M4	8 (19.5)	562.2 ± 792.2	NS	1789.8 ± 3572.8	NS	1.2 ± 0.9	NS	0.6 ± 0.6	NS
	Other subtypes	33 (80.5)	1642.8 ± 1211.9		3293.8 ± 4522.1		4.5 ± 5.2		9.2 ± 11.2	
	M5	9 (30)	1094.2 ± 2003	NS	10925 ± 18246.7	NS	13.3 ± 2.07	0.012	32.2 ± 43	0.021
	Other subtypes	32 (70)	1527 ± 1177		771.3 ± 791.4		1.2 ± 0.5		0.6 ± 0.3	

WBC, white blood cell count; NS not significant.
* Mean ± 0.05 confidence interval.
† Mann–Whitney *U* test.

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